

1652  
#7  
AP

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Art Unit: 1652
	)	
NIELSEN, et al.	)	Examiner: PAK, Y.
	)	
Serial No.: 09/743,419	)	Washington, D.C.
	)	
Filed: November 29, 2001	)	April 22, 2003
	)	
For: METABOLICALLY ENGINEERED )		Docket No.: NIELSEN=5
MICROBIAL CELL COMPRISING)		
A MODIFIED REDOX ACTIVITY)		Confirmation No.: 2282

REQUEST TO VACATE

Commissioner of Patents  
Washington, D.C. 20231

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APR 24 2003  
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S i r :

The restriction requirement mailed March 26, 2003, based on "claims 1-70", should be vacated as it is based on the wrong claims set.

This is a U.S. national stage of PCT/DK99/00398. The international application as filed contained 70 claims. However, in the course of international preliminary examination, the original 70 claim set was replaced with a 59 claim set. The January 10, 2001 transmittal letter enclosed a "Courtesy Copy of the International Preliminary Examination Report with annexes containing claims 1-59 to be substituted for the original claims for examination in this case". We simultaneously filed two preliminary amendments, a main one cancelling claims 2-59, and the supplemental one cancelling claim 1 and adding claims 60-79.

Hence, the office action should have been based on claims 60-79 of the supplemental preliminary amendment, not on claims 1-70 of the PCT application as filed internationally.

USSN - 09/743,419

Copies of the IPE Report, the transmittal letter, and the two preliminary amendments are enclosed herewith.

Respectfully submitted,

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Attorneys for Applicant

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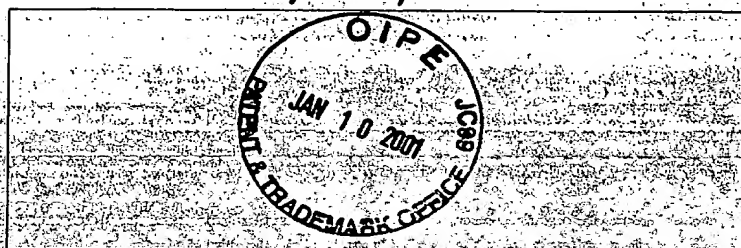
FILED: January 10, 2001  
APPLICANT(S): Jens NIELSEN et al.

DOCKET NO.: NIELSEN S  
BASED ON: PCT/DK99/00398

THE PATENT AND TRADEMARK OFFICE STAMP HEREON  
ACKNOWLEDGES RECEIPT OF THE ABOVE-IDENTIFIED  
APPLICATION, INCLUDING THE FOLLOWING PAPERS:

☒ FEES \$ 990.00 (CH P70-2038)

- ☐ PCT APPLICATION
  - ☐ TRANSMITTAL LETTER REQUEST
  - ☐ FEE CALCULATION SHEET
  - ☐ SPECIFICATION (        pages)
  - ☐ DRAWINGS (        sheets;        figures)
  - ☐ SEQUENCE LISTING ☐ WITH DISK
  - ☐ APPOINTMENT OF AGENT
  - ☐ INVITATION TO CORRECT DEFECTS
  - ☐ REQUEST FOR RECTIFICATION
  - ☐ DEMAND FOR CHAPTER II
  - ☐ ARTICLE 34 AMENDMENTS
  - ☐ RESPONSE TO WRITTEN OPINION
- ☒ U.S. NATIONAL PHASE OF INTERNATIONAL APPLICATION
  - ☒ TRANSMITTAL LETTER
  - ☐ DECLARATION (        pages)
  - ☐ SMALL ENTITY STATEMENT(S) (        pages)
  - ☒ PRELIMINARY AMENDMENT
  - ☐ OTHER



- ☒ SUPPLEMENTAL PRELIMINARY AMENDMENT
- ☐ INFORMATION DISCLOSURE STATEMENT (        pages)
- ☐ FORM 1449 (        pages)
- ☐ PATENTS AND/OR PRINTED PUBLICATIONS
- ☐ SEQUENCE LISTING ☐ WITH DISK
- ☒ COURTESY COPY
- ☒ SPECIFICATION ( 84 pages)
- ☒ DRAWINGS ( 6 sheets; 1-5 figures)
- ☒ 1ST PAGE INTERNATIONAL PUBLICATION
- ☒ INTERNATIONAL SEARCH REPORT
- ☒ IPER ☒ WITH ☐ WITHOUT ANNEXES
- ☐ ENGLISH LANGUAGE TRANS OF SPECIFICATION AS FILED
- ☐ ENGLISH LANGUAGE TRANS OF ANNEXES TO IPER
- ☐ SUBSTITUTE SPECIFICATION


B&N-5

INITIALS: WRP

## PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 29945JL		FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/DK99/00398	International filing date (day/month/year) 12/07/1999	Priority date (day/month/year) 10/07/1998	
International Patent Classification (IPC) or national classification and IPC C12N15/52			
Applicant NIELSEN, Jens et al.			
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 10 sheets, including this cover sheet.</p> <p><input checked="" type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of 6 sheets.</p>			
<p>3. This report contains indications relating to the following items:</p> <p>I <input checked="" type="checkbox"/> Basis of the report</p> <p>II <input checked="" type="checkbox"/> Priority</p> <p>III <input type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</p> <p>IV <input type="checkbox"/> Lack of unity of invention</p> <p>V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</p> <p>VI <input checked="" type="checkbox"/> Certain documents cited</p> <p>VII <input type="checkbox"/> Certain defects in the international application</p> <p>VIII <input checked="" type="checkbox"/> Certain observations on the international application</p>			
Date of submission of the demand 04/02/2000		Date of completion of this report 30.11.2000	
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465		Authorized officer  van Heusden, M  Telephone No. +49 89 2399 8145	



INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT

International application No. PCT/DK99/00398

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*

Description, pages:

1-74 as originally filed

Claims, No.:

1-59 with telefax of 03/11/2000

Drawings, sheets:

1/6-6/6 as originally filed

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).  
☐ the language of publication of the international application (under Rule 48.3(b)).  
☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.  
☐ filed together with the international application in computer readable form.  
☐ furnished subsequently to this Authority in written form.  
☐ furnished subsequently to this Authority in computer readable form.  
☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.  
☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:  
☐ the claims, Nos.:

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/DK99/00398

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**II. Priority**

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

☐ copy of the earlier application whose priority has been claimed.

☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:  
see separate sheet

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

Novelty (N)	Yes:	Claims	18-19, 23-28, 32, 42-47, 49, 51-53, 58-59
	No:	Claims	1-17, 20-22, 29-31, 33-41, 48, 50, 54-57
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-59
Industrial applicability (IA)	Yes:	Claims	1-59
	No:	Claims	

2. Citations and explanations  
see separate sheet

**VI. Certain documents cited**

1. Certain published documents (Rule 70.10)

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/DK99/00398

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and / or

**2. Non-written disclosures (Rule 70.9)**

see separate sheet

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

**Additional remarks to section II:**

The priority claimed in the present application is valid for the present set of claims. Therefore documents D12-D16 do not constitute prior art within the meaning of Rule 64.1 PCT.

**Additional remarks to section V:**

**1. Citations**

The documents mentioned in this IPER are numbered as in the International Search Report (ISR), i.e. D1 corresponds to the first document of the ISR etc.

**2. Novelty (Article 33(2) PCT)**

- 2.1 The present application relates to a eukaryotic microbial cell, more specifically the yeast *Saccharomyces cerevisiae*, that comprises a transhydrogenase activity encoded by *Azotobacter vinelandii*. As a result, glycerol production is increased.
- 2.2 None of documents D1, D2 and D4-D8 discloses a eukaryotic microbial cell comprising an intracellular transhydrogenase activity encoded by a nucleic acid operably linked to an expression signal not natively associated with the nucleic acid.
- 2.3 However, the present application does not satisfy the criterion set forth in Article 33(2) PCT in that the subject matter of claims 1-17, 20-22, 29-31, 33-41, 48, 50 and 54-57 is not novel in view of Document D3.

Document D3 discloses a eukaryotic microbial cell comprising an intracellular transhydrogenase encoded by a nucleic acid operably linked to an expression signal not natively associated with the nucleic acid. However, D3 does not disclose that the expression of said transhydrogenase activity results in an increased production of a metabolite, such as ethanol or glycerol. In fact D3 discloses that both ethanol production and growth rate are decreased.

Interestingly, the microbial cell provided by the present alleged invention also shows reduced ethanol production and growth rate (see Table 2 on p. 60). Glycerol production is not measured in D3 and it is not explicitly disclosed that the production of a metabolite is increased. However, since the microbial cell disclosed in D3 is identical to the eukaryotic microbial cell as defined in claim 1, the increased production of a metabolite is inherent. Thus D3 anticipates the subject matter of claim 1. If the applicant considers that the eukaryotic microbial cell according to claim 1 differs from the microbial cell disclosed in D3, then it appears that an essential technical feature (responsible for the difference between the two cells) is missing from claim 1.

Furthermore, D3 discloses the decreased production of ethanol ('second metabolite'). The microbial cell of D3, in which a heterologous pyridine nucleotide transhydrogenase is over expressed, will inherently have increased levels of coenzymes (NAD, NADP or FAD). Thus D3 anticipates the subject matter of claims 2-13 and 15.

The transhydrogenase as disclosed in D3 falls within the scope of 'a functionally equivalent activity capable of catalysing a transhydrogenase activity'. Thus D3 also anticipates the subject matter of claim 14.

The microbial cell of D3 harbours no endogenous transhydrogenase (Table 1 on p. 669). The transhydrogenase used in D3 is a membrane-bound transhydrogenase from *E. coli*. The microbial cell of D3 is inherently in a broth suitable for culturing or for starting a fermentation. Thus D3 also anticipates the subject matter of claims 16, 17 and 20-22.

The microbial cell of D3 is described to have a reduced growth rate and a retarded ability of fermentation (p. 670, l. 19-20). Thus it appears that said cell produces ethanol, even if at a reduced or slower rate. Therefore the microbial cell of D3 is still suitable for the production of ethanol. Furthermore, as argued above, the cell must inherently show an increased glycerol production (since the microbial cell of D3 is identical to the eukaryotic microbial cell as defined in claim 1) and is thus also suitable for the production of glycerol. Consequently the microbial cell is also suitable for use in a preparation of a drinkable, edible or organoleptic

product. Thus D3 also anticipates the subject matter of claims 29-31 and 33-41.

D3 further describes a method of cultivating a microbial cell according to claim 1 in a suitable growth medium under such conditions that said cell is producing a first metabolite (ethanol) (p. 665, l. 36-39). Thus D3 also anticipates the subject matter of claims 48 and 50.

Furthermore, D3 discloses a method of constructing a microbial cell comprising the steps of operably linking a nucleotide sequence encoding a transhydrogenase activity with an expression signal not natively associated with said nucleotide sequence, and introducing said sequence into a eukaryotic microbial cell (the yeast *S. cerevisiae*), wherein said expression signal results in expression of said operably linked nucleotide sequence. Thus D3 also anticipates the subject matter of claims 54-57.

### 3. Inventive step (Article 33(3) PCT)

- 3.1 The present application does not seem to satisfy the criterion set forth in Article 33(3) PCT because the subject matter of claims 18-19, 23-28, 32, 42-47, 49, 51-53 and 58-59 does not involve an inventive step in view of documents D1-D3.
- 3.2 The closest prior art to evaluate the inventiveness of claims 23-26 and 28 is document D2, which discloses the purification of pyridine transhydrogenase from *A. vinelandii* (p. 528, left column, paragraph 3). The purification of this enzyme is also acknowledged in the description (p. 54, l. 19-21) by reference to Voordouw *et al.*, 1980. Once an enzyme is known and even more so when its purification is disclosed in the prior art, no inventive skill is needed to isolate the DNA sequence encoding the enzyme, to deduce the encoded amino acid sequence or to provide the encoded enzyme by recombinant means.
- 3.3 To transform a eukaryotic microbial cell with the transhydrogenase of *A. vinelandii* does not involve an inventive step in view of document D1 and D3, in combination with document D2. Document D1 discloses the transformation of *E. coli* with the soluble pyrimidine nucleotide transhydrogenase from *Pseudomonas fluorescens*. D2 discloses the high level of similarity between said transhydrogenase from *P.*

*fluorescens* and that from *A. vinelandii*. Therefore it is obvious that the transhydrogenase from *P. fluorescens* can be replaced by that of *A. vinelandii*. D1 discloses that transformation of *E. coli* with a soluble transhydrogenase results in an altered balance/oxidation state of coenzymes such as NAD/NADP or analogs, which in turn results in improved biotransformation, inherently providing increased production of a metabolite. No inventive step is needed to apply this teaching of D1 (relating to bacteria) to yeast. This is even more so in view of the disclosure in D3 in which transformation of a yeast with a transhydrogenase is disclosed. Whereas D3 teaches that transformation with membrane-bound transhydrogenase from *E. coli* does not result in improved fermentation, D3 also states that this may be due to the fact that the transhydrogenase used is membrane-bound and that further suggests that better results may be obtained with water-soluble transhydrogenases (p. 670, last paragraph). The results disclosed in D1 further support this suggestion. Thus, in view of documents D1 and D3, in combination with D2, no inventive step is needed to provide the microbial cell designated TN4 (as defined in claim 14). Also the use of said cell in the production of metabolites or drinkable/edible products does not involve an inventive step. Thus also the subject matter of claims 27, 42-47, 49, 51-53 and 58 does not comply with Article 33(3) PCT.

- 3.4 Claims 18, 19, 32 and 59 do not include any additional matter that could render them inventive as such. These claims would be allowable only in combination with a novel and inventive main claim.

#### 4. Industrial applicability (Article 33(4) PCT)

The subject matter of claims 1-59 is industrially applicable.

#### Additional remarks to section VI:

Document D17, with a publication date of 16.09.99, a filing date of 11.03.99 and a priority date of 11.03.98, does not constitute prior art within the meaning of Rule 64.1(b). However, it appears to disclose features of the present set of claims. No check has been made as to whether the priority of this prior application has been

validly claimed.

**Additional remarks to section VIII:**

The following major objections are raised under **Article 6 PCT** concerning the clarity of the claims:

1. The subject matter of claim 1 is characterized by a result to be achieved, i.e. increased production of a first metabolite. However, the essential technical features required to arrive at such increased production of a metabolite are lacking from claim 1 (see also above under item V.3.4). Thus claims 1-22 and 29-57 and 59 are considered to lack clarity.
2. The wording '**operably linked**' in claims 1-2 lacks clarity in that the IPEA does not understand the exact meaning of this wording. Normally this wording refers to a sequence encoding e.g. an enzyme, which sequence is linked to a control sequence (i.e. a promoter) that controls the expression of said sequence. It is not clear to the IPEA how an enzyme activity is '**operably linked**' to the production of a metabolite. The wording '**operably linked**' is not suitable to define clearly and unambiguously the scope of claims 1-2.
3. The subject matter of claim 17 lacks clarity in that it is not clear to the IPEA what is intended by the wording '**in a natural host organism**'. The same observation applies to the wording '**in a cytoplasm of a natural host organism**' in claim 18. Furthermore, it is noted that the feature of a polypeptide being localized '**in a cytoplasm**' is considered to encompass both membrane-bound and soluble forms of the polypeptide.
4. The subject matter of claims 23, 24 and 28 lacks clarity in that the wording '**or part thereof, including functionally equivalent derivatives**' is not suitable to define clearly and unambiguously the scope of these claims. This wording is open to individual interpretation and thus ambiguous: for example the transhydrogenase of *Pseudomonas fluorescens*, as disclosed in D1, can be considered a derivative (through substitutions/deletions/insertions) being functionally equivalent to the

transhydrogenase represented by SEQ ID NO:1, and would thus anticipate the subject matter of claims 23-25 and 28.

5. The wording '**comparable isolated microbial cell**' in claim 52 lacks clarity in that it is not clear to the IPEA what is meant with this wording.
6. The applicant has shown that the expression of transhydrogenase from *A. vinelandii* in *S. cerevisiae* results in increased glycerol production: according to example 1 of the application, production of glycerol is increased in the TN4 transformant (Table 2 on p. 60), whereas the production of ethanol and the growth rate (biomass) of TN4 is reduced (similar to the results disclosed in D3!). However, the examples do not show any evidence for the increased production of any metabolite other than glycerol. In fact they show a reduced rate of e.g. ethanol production. Therefore it seems that the present set of claims covers a large area of subject matter that is not enabled by the application, for instance the generalisation to an increase of **any** first metabolite, and to a decrease of **any** second metabolite, as well as the generalisation to any intracellular transhydrogenase.

# INTERNATIONAL SEARCH REPORT

Int. Appl. No.  
PCT/DK 99/00398

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/52 C12N15/53 C12N9/00 C12N9/02 C12N9/06  
C12N1/19 C12P7/06 C12P7/20 //(C12N1/19, C12R1:865)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 18909 A (FRENCH CHRISTOPHER EDWARD ;UNIV CAMBRIDGE TECH (GB); BRUCE NEIL CH) 7 May 1998 (1998-05-07)	1,2,6, 8-12,14, 17-19, 28-30, 37,44, 45,47, 51,53, 54,56, 57, 64-66,70 13,35
Y	Note: 82.2% nt sequence identity of SEQ ID NO:1 with SEQ ID NO:1 in 1395 nt overlap, 84.1% aa sequence identity of SEQ ID NO:2 with SEQ ID NO:2 in 464 bp overlap. page 2, line 25 -page 3, line 5 page 3, line 7 -page 4, line 2 page 4, line 9-13 example 3 claims 1-8	

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another, citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

25 January 2000

Date of mailing of the international search report

11/02/2000

Name and mailing address of the ISA

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# INTERNATIONAL SEARCH REPORT

Int. l. Application No  
PCT/DK 99/00398

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VOORDOUW G ET AL: "Why are two different types of pyridine nucleotide transhydrogenase found in living organisms?" EUROPEAN JOURNAL OF BIOCHEMISTRY, DE, BERLIN, vol. 131, no. 3, April 1983 (1983-04), pages 527-533, XP000853531 ISSN: 0014-2956 cited in the application	31-34,36
Y	abstract page 527 page 528, left-hand column, paragraphs 2,3 page 528, right-hand column, paragraph 4 page 529, left-hand column, paragraph 1 figure 1	13,35
X	TANTIRUNGKIJ M ET AL: "Expression of Escherichia coli transhydrogenase genes in Saccharomyces cerevisiae" MICROB. UTIL. RENEWABLE RESOUR. (1996), VOLUME DATE 1995, vol. 9, pages 664-672, XP000866358  the whole document page 670, paragraph 3	1-12, 14-19, 28-30, 37,38, 41,42, 44-62, 64-68,70
X	EP 0 733 712 A (AJINOMOTO KK) 25 September 1996 (1996-09-25) cited in the application   page 2, line 48-55 page 3, line 3-5,11-45 examples 1-4 claims 1-8	1,2,6, 8-12,19, 28-30, 37,44, 45, 47-49, 51,53, 54,56, 57, 64-66,70
X	WO 96 41888 A (INST NAT RECH AGRONOMIQUE IN ;DEQUIN SYLVIE (FR); BARRE PIERRE (FR) 27 December 1996 (1996-12-27)  the whole document example 2 claims 1,10	1-9,19, 28-30, 37,38, 42, 44-61, 65-68,70

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# INTERNATIONAL SEARCH REPORT

Int. l. Application No  
PCT/DK 99/00398

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DE VOS W M: "Metabolic engineering of sugar catabolism in lactic acid bacteria;" ANTONIE LEEUWENHOEK J. MICROBIOL., vol. 70, no. 2-4, October 1996 (1996-10), pages 223-242, XP000866282</p> <p>the whole document page 225, left-hand column, paragraph 2 -page 231, left-hand column, paragraph 1 page 236, left-hand column, paragraph 2 -page 238, right-hand column, paragraph 1</p>	<p>1-6, 8-11, 20, 23, 25, 28-30, 37, 39, 43-45, 47-49, 51, 53, 54, 56-58, 63, 65, 66, 70</p>
X	<p>LOPEZ DE FELIPE, F. ET AL: "The role of NADH -oxidation in acetoin and diacetyl production from glucose in Lactococcus lactis subsp. lactis MG1363" FEMS MICROBIOLOGY LETTERS, vol. 156, no. 1, 1 November 1997 (1997-11-01), pages 15-19, XP000866569</p> <p>abstract page 19, left-hand column, line 23-29</p>	<p>1-6, 8-11, 28-30, 37, 39, 43-45, 47-49, 51, 53, 54, 56-58, 63, 65, 66, 70</p>
X	<p>EP 0 462 674 A (GIST BROCADES NV) 27 December 1991 (1991-12-27)</p> <p>the whole document claims 18, 19</p>	<p>1, 6, 28-30, 37, 40, 44, 45, 47, 48, 51, 53, 54, 56-58, 65, 66</p>
A	<p>VAN DIJKEN, J.P. ET AL: "Redox balances in the metabolism of sugars by yeasts." FEMS MICROBIOLOGY REVIEWS, vol. 32, no. 3-4, 1986, pages 199-224, XP000866552</p> <p>the whole document page 199-203 page 217 page 219-222</p>	<p>1-19, 37, 38, 41, 42, 44-62</p>

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# INTERNATIONAL SEARCH REPORT

Int. l. Application No  
PCT/DK 99/00398

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VAN RIEL N A W ET AL.: "A structured, minimal parameter model of the central nitrogen metabolism in <i>Saccharomyces cerevisiae</i> : the prediction of the behaviour of mutants" JOURNAL OF THEORETICAL BIOLOGY, vol. 191, no. 4, 21 April 1998 (1998-04-21), pages 397-414, XP000852821 abstract page 398; figure 1 page 404; figure 4 page 409, left-hand column, line 5-18 page 410, right-hand column, line 7-11 page 411, left-hand column, line 2-6	20-27
A	AVENDANO A ET AL: "GDH3 encodes a glutamate dehydrogenase isozyme, a previously unrecognized route for glutamate biosynthesis in <i>Sacharomyces cerevisiae</i> " JOURNAL OF BACTERIOLOGY, US, WASHINGTON, DC, vol. 179, no. 17, September 1997 (1997-09), page 5594-5597 XP002085996 ISSN: 0021-9193 abstract	21,22, 24,26
P,X	DATABASE EMPRO 'Online! EMBL ID AF159108, AC AF159108, 29 June 1999 (1999-06-29) BOONSTRA B ET AL.: "Azotobacter vinelandii soluble pyridine nucleotide transhydrogenase (sth) gene, complete cds" XP002128388 Note: 99.6% nt-sequence identity with SEQ ID NO:1 in 1395 nt overlap. the whole document	1,2,6, 8-15, 17-19, 28-37, 64-66,70
P,X	ANDERLUND M ET AL.: "Expression of the <i>Escherichia coli</i> pntA and pntB genes, encoding nicotinamide nucleotide transhydrogenase, in <i>Saccharomyces cerevisiae</i> and its effect on product formation during anaerobic fermentation" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 65, no. 6, June 1999 (1999-06), pages 2333-2340, XP000856677 the whole document page 334, left-hand column, line 10-20	1-12, 14-16, 18,19, 28-30, 37,38, 41,42, 44-62, 64-68,70

-/--

# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/DK 99/00398

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>WO 99 28480 A (GENECOR INTERNATIONAL INC ;DU PONT (US); NAIR RAMESH V (US); PAYNE) 10 June 1999 (1999-06-10)</p> <p>the whole document examples 1-10 claims 1-10</p>	<p>1-9, 19, 20, 25, 27-30, 37-40, 42, 44-61, 65-68, 70</p>
P,X	<p>WO 98 54337 A (HANSENS LAB ;NILSSON DAN (DK); KRINGELUM BOERGE (DK)) 3 December 1998 (1998-12-03)</p> <p>abstract page 10, line 2 -page 12, line 7 claims 14-16, 30-32, 37-41</p>	<p>1-6, 8-11, 28-30, 37, 39, 43-45, 47-49, 51, 53, 54, 56-58, 63, 65, 66, 70</p>
P,X	<p>LOPEZ DE FELIPE F ET AL.: "Cofactor engineering: a novel approach to metabolic engineering in Lactococcus lactis by controlled expression of NADH oxidase" JOURNAL OF BACTERIOLOGY, vol. 180, no. 15, August 1998 (1998-08), pages 3804-3808, XP002128387 cited in the application</p> <p>abstract</p>	<p>1-6, 8-11, 19, 28-30, 37, 39, 43-45, 47-49, 51, 53, 54, 56-58, 63, 65, 66, 70</p>
E	<p>WO 99 46363 A (ARISTIDOU ARISTOS ;VALTION TEKNILLINEN (FI); RICHARD PETER (FI); T) 16 September 1999 (1999-09-16)</p> <p>the whole document examples 1, 8, 9, 13, 14</p>	<p>1-11, 19, 20, 23, 28-30, 37, 38, 41, 42, 44-62, 65-68, 70</p>

## PATENT CLAIMS

1. Eukaryotic microbial cell comprising an intracellular transhydrogenase activity encoded by a nucleic acid operably linked to an expression signal not natively associated with the nucleic acid, wherein the expression of the intracellular transhydrogenase activity is operably linked to an increased production of a first metabolite.
2. Microbial cell according to claim 1 wherein the expression of the intracellular transhydrogenase activity is further operably linked to a decreased production of the second metabolite.
3. Microbial cell according to claim 1, said cell being selected from the group consisting of a fungal cell and a yeast cell.
4. Microbial cell according to claim 1, said cell being a yeast cell.
5. Microbial cell according to claim 1 wherein the expression of said intracellular transhydrogenase results in an increased level of at least one intracellular coenzyme in its oxidised or reduced form.
6. Microbial cell according to claim 1 wherein said coenzyme in its oxidised/reduced form is NAD/NADH.
7. Microbial cell according to claim 1 wherein said coenzyme in its oxidised/reduced form is NADP/NADPH.
8. Microbial cell according to claim 1 wherein said coenzyme in its oxidised/reduced form is FAD/FADH<sub>2</sub>.
9. Microbial cell according to claim 1 wherein the expression of said intracellular transhydrogenase results in an increased level of at least one intracellular redox system.
10. Microbial cell according to claim 9 wherein said redox system is NAD/NADH.

11. Microbial cell according to claim 9 wherein said redox system is NADP/NADPH.
12. Microbial cell according to claim 9 wherein said redox system is FAD/FADH<sub>2</sub>.
- 5 13. Microbial cell according to claim 1 wherein said intracellular transhydrogenase is a pyridine nucleotide transhydrogenase activity.
- 10 14. Microbial cell according to claim 13 wherein said transhydrogenase activity is that encoded by *CTH* of *Azotobacter vinelandii* as harboured by *Saccharomyces cerevisiae* TN4 deposited under DSM Accession Number 12267, or a functionally equivalent activity capable of catalysing a transhydrogenase activity.
- 15 15. Microbial cell according to claim 13 wherein said pyridine nucleotide transhydrogenase activity is heterologous to said cell.
- 16 16. Microbial cell according to claim 1 wherein said cell harbours no endogenous transhydrogenase activity.
- 20 17. Microbial cell according to claim 14 or 15 wherein said transhydrogenase activity is exhibited by a polypeptide which is membrane-bound in a natural host organism.
- 25 18. Microbial cell according to claim 14 or 15 wherein said transhydrogenase activity is exhibited by a polypeptide which is located in a cytoplasm of a natural host organism.
- 30 19. Microbial cell according to any of the previous claims in the form of a frozen or freeze-dried preparation such as a reconstitutable lyophilisate.
- 35 20. Composition comprising the microbial cell according to any of the previous claims and a physiologically acceptable carrier.
21. Composition according to claim 20 wherein said carrier is a water-based liquid and preferably a broth suitable for culturing said microbial cell.

22. Composition according to claims 20 or 21, said composition being a fermentation starter culture.
- 5 23. Nucleotide sequence encoding a transhydrogenase enzyme activity, said sequence comprising SEQ ID NO:1, or part thereof, including functionally equivalent derivatives.
- 10 24. Nucleotide sequence according to claim 23 wherein the functionally equivalent derivatives comprise conservative nucleotide substitutions and/or nucleotide deletions and/or nucleotide insertions.
25. Recombinant DNA-replicon in the form of a vector comprising the nucleotide sequence according to claim 23 or 24.
- 15 26. Recombinant DNA-replicon according to claim 25, said replicon being harboured by *Saccharomyces cerevisiae* TN4 deposited under DSM Accession Number 12267.
- 20 27. Microbial cell according to any of claims 1 to 19 transformed with the nucleotide sequence of claim 23 to 24 or the vector of claim 25 or 26.
- 25 28. Amino acid sequence encoded by the nucleotide sequence of claim 23 or 24, said sequence comprising SEQ ID NO:2, or part thereof, including functionally equivalent derivatives.
29. Microbial cell according to any of claims 1 to 19 and 27 or composition according to any of claims 20 to 22 for use in a production of a first metabolite.
- 30 30. Microbial cell according to claim 29, said cell being a yeast cell.
31. Microbial cell according to claim 30, said cell being a *Saccharomyces cerevisiae* cell.
- 35 32. Microbial cell according to claim 29, said cell being selected from the group consisting of a *Penicillium* cell and an *Aspergillus* cell.

33. Microbial cell according to claim 29 wherein said first metabolite is ethanol.

34. Microbial cell according to claim 29 wherein said first metabolite is glycerol.

35. Microbial cell according to any of claims 1 to 19 and 27 or composition according to any of claims 20 to 22 for use in a production of a first metabolite and a second metabolite.

36. Microbial cell according to claim 35 for use in an increased production of a first metabolite and a decreased production of a second metabolite.

37. Microbial cell according to claim 36 wherein said first metabolite is glycerol and said second metabolite is ethanol.

38. Microbial cell according to any of claims 1 to 19 and 27, or composition according to any of claims 20 to 22, for use in a preparation of a drinkable or an edible product.

39. Microbial cell according to any of claims 1 to 19 and 27, or composition according to any of claims 20 to 22, for use in a production of a first metabolite for use in a drinkable or an edible product.

40. Microbial cell according to claim 39, wherein said first metabolite has and/or provides a desirable organoleptic quality to said product.

41. Microbial cell according to claim 39, said first metabolite being selected from the group consisting of ethanol and glycerol.

42. Use of the microbial cell according to any of claims 1 to 19 and 27, or composition according to any of claims 20 to 22, in the production of a first metabolite.

43. Use according to claim 42 wherein said first metabolite is selected from the group consisting of ethanol and glycerol.

44. Use of the microbial cell according to any of claims 1 to 19 and 27, or composition according to any of claims 20 to 22, in the production of a first and a second metabolite.
5. 45. Use according to claim 44 in an increased production of a first metabolite and a decreased production of a second metabolite.
46. Use according to claim 45 wherein said first metabolite is glycerol and said second metabolite is ethanol.
- 10 47. Use of a microbial cell according to any of claims 1 to 19 and 27, or composition according to any of claims 20 to 22, in a preparation of a drinkable or edible product.
- 15 48. Method of producing a first metabolite comprising the steps of
- i) cultivating microbial cell according to any of claims 1 to 19 and 21 in a suitable growth medium and under such conditions that said microbial cell is producing a first metabolite,
- 20 and optionally
- ii) isolating said first metabolite in a suitable form,
- 25 and further optionally
- iii) purifying said isolated first metabolite.
- 30 49. Method of claim 48 wherein said production of said first metabolite being substantially increased as compared to the production of the metabolite in a comparable wild-type cell or a comparable isolated microbial cell.
50. Method of claim 48 wherein said microbial cell is a yeast cell.
- 35 51. Method of claim 48 wherein said first metabolite is glycerol.

52. Method of claim 48, said yeast cell further producing a second metabolite, the production of said second metabolite being substantially decreased as compared to the production of said second metabolite in a comparable wild-type cell or a comparable isolated microbial cell.

53. Method of claim 52 wherein said second metabolite is ethanol.

54. Method of constructing a microbial cell comprising the steps of

- i) operably linking a nucleotide sequence encoding a transhydrogenase activity with an expression signal not natively associated with said nucleotide sequence, and
- ii) introducing said operably linked nucleotide sequence obtained under i) into a eukaryotic microbial cell wherein said expression signal results in expression of said operably linked nucleotide sequence.

55. Method of claim 54, said microbial cell being selected from the group consisting of a fungal cell and a yeast cell.

56. Method of claim 55, said microbial cell being a yeast cell.

57. Method of claim 56, said yeast cell being a *Saccharomyces cerevisiae* cell.

58. Method of claim 57 wherein said cell is *Saccharomyces cerevisiae* TN4 deposited under DSM Accession Number 12267.

59. Method according to any of claims 54 to 58, said method comprising a further step of freezing or freeze-drying the microbial cell in the preparation of a reconstitutable lyophilisate.

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER <b>NIELSEN 5</b>
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>		U.S. APPLICATION NO. (If known, see 37 CFR 1.5)
INTERNATIONAL APPLICATION NO. <b>PCT/DK99/00398</b>	INTERNATIONAL FILING DATE <b>12 July 1999</b>	PRIORITY CLAIMED <b>10 July 1998</b>
TITLE OF INVENTION <b>METABOLICALLY ENGINEERED MICROBIAL CELL COMPRISING A MODIFIED REDOX ACTIVITY</b>		
APPLICANT(S) FOR DO/EO/US <b>Jens NIELSEN et al.</b>		
<p>Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:</p> <ol style="list-style-type: none"> <li><input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li><input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li><input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</li> <li><input checked="" type="checkbox"/> The US has been elected in a Demand by the expiration of 19 months from the priority date (PCT Article 31).</li> <li><input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))             <ol style="list-style-type: none"> <li><input type="checkbox"/> is attached hereto (required only if not transmitted by the International Bureau).</li> <li><input checked="" type="checkbox"/> has been communicated by the International Bureau.</li> <li><input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> </li> <li><input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</li> <li><input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))             <ol style="list-style-type: none"> <li><input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li><input type="checkbox"/> have been communicated by the International Bureau.</li> <li><input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li><input checked="" type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li><input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li> <li><input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</li> <li><input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol> <p>Items 11. to 16. below concern document(s) or information included:</p> <ol style="list-style-type: none"> <li><input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</li> <li><input type="checkbox"/> An Assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li><input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment. <input checked="" type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</li> <li><input type="checkbox"/> A substitute specification.</li> <li><input type="checkbox"/> A change of power of attorney and/or address letter.</li> <li><input checked="" type="checkbox"/> Other items or information:             <ul style="list-style-type: none"> <li><input checked="" type="checkbox"/> Courtesy copy of the International Application as filed.</li> <li><input checked="" type="checkbox"/> Courtesy copy of the first page of the International Publication (WO 00/03021).</li> <li><input checked="" type="checkbox"/> Courtesy copy of the International Preliminary Examination Report with annexes containing claims 1-59 to be substituted for the original claims for examination in this case.</li> <li><input checked="" type="checkbox"/> Formal drawings, 6 sheets, Figures 1-5.</li> <li><input checked="" type="checkbox"/> Courtesy Copy of the International Search Report.</li> <li><input checked="" type="checkbox"/> Sequence Listing</li> </ul> </li> </ol>		

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	International Application No. <b>PCT/DK99/00398</b>	Attorney's Docket No. <b>NIELSEN 5</b>
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<p>17. [xx] The following fees are submitted:</p> <p><b>BASIC NATIONAL FEE (37 CFR 1.492 (a)(1) –(5):</b>  Neither international preliminary examination fee (37 CFR 1.482)  nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO  and International Search Report not prepared by the EPO or JPO.....\$1000.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to  USPTO but International Search Report prepared by the EPO or JPO.....\$860.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but  international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$710.00</p> <p>International preliminary examination fee paid to USPTO (37 CFR 1.482)  but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$690.00</p> <p>International preliminary examination fee paid to USPTO (37 CFR 1.482)  and all claims satisfied provisions of PCT Article 33(1)-(4).....\$100.00</p> <p style="text-align: center;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b></p> <p>Surcharge of \$130.00 for furnishing the oath or declaration later than [ ] 20 [X] 30  months from the earliest claimed priority date (37 CFR 1.492(e)).</p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%;">Claims as Originally Presented</th> <th style="width: 15%;">Number Filed</th> <th style="width: 15%;">Number Extra</th> <th style="width: 15%;">Rate</th> <th style="width: 10%;"></th> <th style="width: 15%;"></th> </tr> <tr> <td>Total Claims</td> <td>1 - 20</td> <td></td> <td>X \$18.00</td> <td>\$</td> <td></td> </tr> <tr> <td>Independent Claims</td> <td>1 - 3</td> <td></td> <td>X \$80.00</td> <td>\$</td> <td></td> </tr> <tr> <td colspan="3">Multiple Dependent Claims (if applicable)</td> <td>+\$270.00</td> <td>\$</td> <td></td> </tr> </table> <p style="text-align: center;"><b>TOTAL OF ABOVE CALCULATIONS =</b></p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%;">Claims After Post Filing Prel. Amend</th> <th style="width: 15%;">Number Filed</th> <th style="width: 15%;">Number Extra</th> <th style="width: 15%;">Rate</th> <th style="width: 10%;"></th> <th style="width: 15%;"></th> </tr> <tr> <td>Total Claims</td> <td>20 - 20</td> <td></td> <td>X \$18.00</td> <td>\$</td> <td></td> </tr> <tr> <td>Independent Claims</td> <td>1 - 3</td> <td></td> <td>X \$78.00</td> <td>\$</td> <td></td> </tr> </table> <p style="text-align: center;"><b>TOTAL OF ABOVE CALCULATIONS =</b></p> <p>Reduction of ¼ for filing by small entity, if applicable. Applicant claims small entity  status. See 37 CFR 1.27.</p> <p style="text-align: center;"><b>SUBTOTAL =</b></p> <p>Processing fee of \$130.00 for furnishing the English translation later than [ ] 20 [ ] 30  months from the earliest claimed priority date (37 CFR 1.492(f)).</p> <p style="text-align: center;"><b>TOTAL NATIONAL FEE =</b></p> <p>Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be  accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +</p> <p style="text-align: center;"><b>TOTAL FEES ENCLOSED =</b></p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 60%;"></td> <td style="width: 20%; text-align: right;">Amount to be:</td> <td style="width: 20%; text-align: right;">\$</td> </tr> <tr> <td></td> <td style="text-align: right;">refunded</td> <td></td> </tr> <tr> <td></td> <td style="text-align: right;">charged</td> <td style="text-align: right;">\$</td> </tr> </table>	Claims as Originally Presented	Number Filed	Number Extra	Rate			Total Claims	1 - 20		X \$18.00	\$		Independent Claims	1 - 3		X \$80.00	\$		Multiple Dependent Claims (if applicable)			+\$270.00	\$		Claims After Post Filing Prel. Amend	Number Filed	Number Extra	Rate			Total Claims	20 - 20		X \$18.00	\$		Independent Claims	1 - 3		X \$78.00	\$			Amount to be:	\$		refunded			charged	\$	<b>CALCULATIONS PTO USE ONLY</b>
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	charged	\$																																																		

a. [ ] A check in the amount of \$ \_\_\_\_\_ to cover the above fees is enclosed.

b. [X] Credit Card Payment Form (PTO-2038), authorizing payment in the amount of \$ 990.00, is attached.

c. [ ] Please charge my Deposit Account No. 02-4035 in the amount of \$ \_\_\_\_\_ to cover the above fees.  
A duplicate copy of this sheet is enclosed.

d. [XX] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment  
to Deposit Account No. 02-4035. A duplicate copy of this sheet is enclosed.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or  
(b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

**BROWDY AND NEIMARK, P.L.L.C.**  
**624 NINTH STREET, N.W., SUITE 300**  
**WASHINGTON, D.C. 20001**  
**TEL: (202) 628-5197**  
**FAX: (202) 737-3528**  
**Date of this submission: January 10, 2001**

\_\_\_\_\_  
SIGNATURE  
**Iver P. Cooper**  
\_\_\_\_\_  
NAME  
**28,005**  
\_\_\_\_\_  
REGISTRATION NUMBER

Form PTO-1390 (as slightly revised by Browdy and Neimark)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Art Unit:
Jens NIELSEN et al.	)	
	)	
	)	
IA No.: PCT/DK99/00398	)	
	)	Washington, D.C.
IA Filed: 12 July 1999	)	
	)	
U.S. App. No.:	)	
(Not Yet Assigned)	)	
	)	January 10, 2001
National Filing Date:	)	
(Not Yet Received)	)	
	)	
For: METABOLICALLY ENGINEERED...	)	Docket No.: NIELSEN 5

PRELIMINARY AMENDMENT

Honorable Commissioner of Patents and Trademarks  
Washington, D.C. 20231

Sir:

Contemporaneous with the filing of this case and  
prior to calculation of the filing fee, kindly amend as  
follows:

IN THE SPECIFICATION

After the title please insert the following  
paragraph:

—The present application is the national stage  
under 35 U.S.C. 371 of PCT/DK99/00398, filed 12 July 1999.--

IN THE CLAIMS

Delete claims 2-59.

REMARKS

The above amendment to the specification is being made to insert reference to the PCT application of which the present case is a U.S. national stage. The above amendments to the claims are being made in order to eliminate claims, for the purpose of reducing the filing fee. Please enter this amendment prior to calculation of the filing fee in this case.

Favorable consideration and allowance are earnestly solicited.

Respectfully submitted,  
BROWDY AND NEIMARK, P.L.L.C.  
Attorneys for Applicant

By: 

Iver P. Cooper  
Registration No. 28,005

IPC:wrđ  
Telephone No.: (202) 628-5197  
Facsimile No.: (202) 737-3528

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Art Unit:
Jens NIELSEN et al.	)	
	)	
	)	
IA No.: PCT/DK99/00398	)	
	)	Washington, D.C.
IA Filed: 12 July 1999	)	
	)	
U.S. App. No.:	)	
(Not Yet Assigned)	)	
	)	January 10, 2001
National Filing Date:	)	
(Not Yet Received)	)	
	)	
For: METABOLICALLY ENGINEERED...	)	Docket No.: NIELSEN 5

SUPPLEMENTAL PRELIMINARY AMENDMENT

Honorable Commissioner of Patents and Trademarks  
Washington, D.C. 20231

Sir:

Prior to examination upon the merits, kindly amend  
as follows:

Delete claim 1, and insert the following claims:

—60. Eukaryotic microbial cell comprising an  
intracellular transhydrogenase activity encoded by a nucleic  
acid operably linked to an expression signal not natively  
associated with the nucleic acid,

wherein the intracellular transhydrogenase is a cytoplasmic  
transhydrogenase when the eukaryotic microbial cell is a  
yeast cell, and

wherein the expression of the intracellular transhydrogenase  
activity results in  
an increased production of a first metabolite.

61. Microbial cell according to claim 60 wherein the expression of the intracellular transhydrogenase activity further results in a decreased production of the second metabolite.

62. Microbial cell according to claim 60, said cell being selected from the group consisting of a fungal cell and a yeast cell.

63. Microbial cell according to claim 60, said cell being a yeast cell.

64. Microbial cell according to claim 60 wherein the expression of said intracellular transhydrogenase results in an increased level of at least one intracellular coenzyme in its oxidised or reduced form.

65. Microbial cell according to claim 64 wherein said coenzyme in its oxidised/reduced form is NAD/NADH.

66. Microbial cell according to claim 64 wherein said coenzyme in its oxidised/reduced form is NADP/NADPH.

67. Microbial cell according to claim 64 wherein said coenzyme in its oxidised/reduced form is FAD/FADH<sub>2</sub>.

68. Microbial cell according to claim 60 wherein said intracellular transhydrogenase is a pyridine nucleotide transhydrogenase.

69. Microbial cell according to claim 68 wherein said transhydrogenase activity is that encoded by CTH of *Azotobacter vinelandii* as harboured by *Saccharomyces*

cerevisiae TN4 deposited under DSM Accession Number 12267, or a function ally equivalent activity capable of catalysing a transhydrogenase.

70. Microbial cell according to claim 68 wherein said pyridine nucleotide trans 10 hydrogenase is heterologous to said cell.

71. Microbial cell according to claim 68 wherein said cell harbours no endogenous transhydrogenase.

72. Composition comprising the microbial cell according to claim 60 and a physiologically acceptable carrier.

73. Microbial cell according to claim 60 wherein said first metabolite is ethanol.

74. Microbial cell according to claim 60 wherein said first metabolite is glycerol.

75. Microbial cell according to claim 60 for use in a preparation of a drinkable or an edible product.

76. Method of producing a first metabolite comprising the steps of

i) cultivating microbial cell according to claim 60 in a suitable growth medium and under such conditions that said microbial cell is producing a first metabolite, 30 and optionally

ii) isolating said first metabolite in a suitable form,

and further optionally purifying said isolated first metabolite.

77. Method of claim 76, said yeast cell further producing a second metabolite, the production of said second metabolite being substantially decreased as compared to the production of said second metabolite in a comparable wild-type cell or a comparable isolated microbial cell.

78. Method of claim 77 wherein said first metabolite is ethanol and said second metabolite is glycerol.

79. Method of constructing a microbial cell according to claim 60 comprising the steps of

i) operably linking a nucleotide sequence encoding a transhydrogenase activity with an expression signal not natively associated with said nucleotide sequence, and

ii) introducing said operably linked nucleotide sequence obtained

under i) into a eukaryotic microbial cell, wherein said expression signal results in expression of said operably linked nucleotide sequence

wherein the intracellular transhydrogenase is a cytoplasmic transhydrogenase when the eukaryotic microbial cell is a yeast cell.—

#### REMARKS

Claims 60-79 presently appear in this case.

The above amendments to the claims are being made in order to add new claims and to restore at least partly the varying scope of claims which was eliminated by the elimination of multiple dependencies in the claims.

Favorable consideration is earnestly solicited.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.  
Attorneys for Applicant

By: 

Iver P. Cooper  
Registration No. 28,005

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☐ **FADED TEXT OR DRAWING**

☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☐ **SKEWED/SLANTED IMAGES**

☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☐ **GRAY SCALE DOCUMENTS**

☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☒ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** \_\_\_\_\_

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